

Antiproliferative effect of the *Ginkgo biloba* extract is associated with the enhancement of cytochrome P450 1B1 expression in estrogen receptor-negative breast cancer cells

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Abstract. *Ginkgo biloba* is a dioecious tree and its extract is a complex mixture that has been used for thousands of years to treat a variety of ailments in traditional Chinese medicine. The aim of this study was to present our observations on the inhibitory effects of different *Ginkgo biloba* extracts on human breast cancer cell proliferation and growth. Our results demonstrated that treatment of MCF-7 and MDA-MB-231 human breast cancer cells with *Ginkgo biloba* leaves and ginkgo fruit extract inhibited cell proliferation. It was also observed that this inhibition was accompanied by the enhancement of cytochrome P450 (CYP) 1B1 expression in MDA-MB-231 cells. In addition, treatment with ginkgo fruit extract resulted in a higher CYP1B1 expression in MDA-MB-231 cells compared to treatment with the *Ginkgo biloba* leaves extract. Our results suggested that the inhibitory effects of the *Ginkgo biloba* extract on estrogen receptor-negative breast cancer proliferation and the induction of CYP1B1 expression may be exerted through an alternative pathway, independent of the estrogen receptor or the aryl hydrocarbon receptor pathway.

Introduction

Ginkgo biloba is a dioecious tree and has been used for thousands of years to treat a variety of ailments in traditional Chinese medicine (1). The *Ginkgo biloba* extract is a complex mixture that mainly contains flavonoids (primarily quercetin, kaempferol and isorhamnetin) and terpene lactones (ginkgolides and bilobalide) (2). Extensive investigation of the main bioactive constituents of the *Ginkgo biloba* extract revealed several important pharmacological effects. It was previously reported that the *Ginkgo biloba* extract exerts an antioxidant effect by

scavenging reactive oxygen species (3), reduces platelet aggregation and exhibits neuroprotective properties (4). Previous studies demonstrated the potential benefits of the *Ginkgo biloba* extract in the treatment of Alzheimer's disease (5), learning and memory deficits (6), cerebrovascular diseases (7), cardiovascular diseases (8), climacteric vasomotor symptoms and postmenopausal syndrome (9,10). It was also demonstrated that the *Ginkgo biloba* extract possesses antitumor properties (11), may induce cancer cell apoptosis and differentiation and inhibit the progression of human colon cancer (12), hepatocellular carcinoma (13), pancreatic (14) and gastric cancer (15).

Cytochrome P450 (CYP) is a type of heme-thiolate protein that is ubiquitously found in the biosphere. This enzyme family is crucial in several biological processes, such as the oxidative metabolism of exogenous and endogenous organic chemicals, the biological transformation of drugs or xenobiotics, the metabolism of chemical carcinogens and the biosynthesis of physiologically crucial compounds, such as steroids and fatty acids (16). Human CYPs are associated with a number of diseases, such as hypertension, diabetes, obesity and hepatic, infectious and inflammatory diseases (17,18). Members of the CYP family have been identified in healthy and cancerous extrahepatic tissue, such as breast cancer cells, and are associated with tumor development and progression (19-24).

Three types of CYP1 enzymes are expressed in humans, CYP1A1, CYP1A2 and CYP1B1. The members of this family are under the transcriptional regulation of the aryl hydrocarbon receptor (AhR) and are known to activate pro-carcinogens, such as polycyclic aromatic hydrocarbons (PAHs) (25). All the members of this family are expressed in extrahepatic tissues. However, CYP1B1 is uniquely overexpressed in a wide range of human cancers of different histogenetic types compared to normal tissues (26-28). Several studies reported that the *Ginkgo biloba* extract exhibits differential induction of CYPs (29-31). The aim of this study was to assess whether the *Ginkgo biloba* extract induces CYP1B1 expression and affects the proliferation of MCF-7 and MDA-MB-231 human breast cancer cells.

Materials and methods

Ginkgo biloba and *Ginkgo biloba* extract. The *Ginkgo biloba* leaves and ginkgo fruit were obtained from the Shenyang

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Agricultural University, without prior exposure to chemical pesticides. This study was conducted in accordance with the University's ethic regulations concerning the use of human related materials in scientific research and with the approval of the ethics committee of Shenyang Agricultural University.

High-quality *Ginkgo biloba* leaves and ginkgo fruit were used for the preparation of an aqueous extract. *Ginkgo biloba* leaves and ginkgo fruit (50 g) were cut and minced separately and 100 ml cold water was added to suspend the minced *Ginkgo biloba* mixture. The mixture was maintained at 80°C for 40 min and was then subjected to negative pressure filtration, followed by the addition of distilled water to the clear supernatant to a final volume of 100 ml. The supernatant was then filtered through a 0.45- μ m membrane filter into a sterile collection bottle and was kept at 2–8°C in a refrigerator as a final extract for subsequent experimental use.

Cell lines and cell culture. The MCF-7 and MDA-MB-231 human breast cancer cell lines were purchased from the cell bank of the Chinese Academy of Sciences. MCF-7 and MDA-MB-231 cells were removed from the liquid nitrogen and preheated for 1 min at 37°C. The cells were then transferred to a 25-cm² cell culture vessel, followed by the addition of 10–15 ml RPMI-1640 cell culture medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. The cells were then cultured at 37°C in an atmosphere of 5% CO₂ in a tissue culture apparatus. The cell culture medium was changed after culture for 12 h. After growing to 80–90% confluency, the cells were harvested and reseeded for the treatment assay.

Experimental treatments. For the *Ginkgo biloba* extract treatment assay, MCF-7 and MDA-MB-231 cells were divided into three treatment groups: the control group, the *Ginkgo biloba* leaves extract group and the ginkgo fruit extract group. Cells (1–1.5 $\times 10^6$ MCF-7 or 4–5 $\times 10^6$ MDA-MB-231) were plated in 10–15 ml of RPMI-1640 cell culture medium supplemented with 10% FBS and 1% antibiotics. The MCF-7 cells were then incubated with 500 μ l extract of *Ginkgo biloba* leaves as the first group and 500 μ l extract of ginkgo fruit as the second group. Cells without the addition of *Ginkgo biloba* extract served as the control group. MDA-MB-231 cells were treated with *Ginkgo biloba* extract at the same dose as MCF-7 cells. Cell morphology was observed after 48 h under a confocal laser scanning microscope. The cells of each group were then collected and counted and RNA was extracted.

Qualitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and the first-strand complementary DNA (cDNA) was synthesized according to the manufacturer's instructions, using 1 μ g total RNA with a random primer and the Moloney murine leukemia virus reverse transcriptase, RNase H minus [M-MLV RTase (RNase H⁻)] (GeneCopoeia, Inc., Rockville, MD, USA). The first-strand cDNA was stored at –20°C for later use.

The PCR primers for CYP1B1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were intron-spanning primer sequences of CYP1B1 and GAPDH and were as follows:

CYP1B1: sense, 5'-GGCTGGATTTGGAGAACGTA-3' and antisense, 5'-GTTGATGAGGCCATCCTTGT-3'; GAPDH: sense, 5'-GGATTGCTCGTATTGGG-3' and antisense, 5'-GGAAGATGGTGATGGGATT-3'. The size of the PCR products was 419 and 205 bp, respectively.

The PCR reactions were performed on a Bio-Rad S1000 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR amplification was performed in a total reaction volume of 25 μ l, containing 1 μ l of cDNA sample, 10 pM of each primer, 2.5 mM of deoxyribonucleotide, 10X EasyTaq buffer and 5 units of EasyTaq DNA polymerase (TransGen Biotech, Beijing, China). The cycling parameters were: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and a final extension at 72°C for 30 sec. The PCR products were analyzed on 1.2% (w/v) agarose gels containing 0.5 μ g/ml ethidium bromide and were visualized under UV light.

Quantitative PCR (qPCR). qPCR was performed to detect CYP1B1 gene expression. The 20- μ l reaction mixture contained 4.6 μ l diethylpyrocarbonate-H₂O, 1.0 μ l cDNA, 2.0 μ l (10 μ M) of each primer, 10 μ l 2X All-in-One qPCR Mix and 0.4 μ l 50X ROX Reference Dye (GeneCopoeia). The thermal cycle program for PCR was as follows: an initial step at 94°C for 10 min, followed by 40 cycles of PCR at 94°C for 10 sec, at 56°C for 20 sec and at 72°C for 20 sec. The fluorescence signal was digitally collected after each cycle of 72°C for 20 sec. Following PCR amplification, the samples were subjected to a temperature ramp with continuous fluorescence monitoring for melting curve analysis. LightCycler 480 analysis software (Roche Light Cycler 480, Hoffmann-La Roche, Ltd., Basel, Switzerland) was used to obtain the Ct values. The 2^{– $\Delta\Delta$ Ct} method (32) was used to analyze the relative expression of CYP1B1 in MCF-7 or MDA-MB-231 cells treated with *Ginkgo biloba* leaves extract or ginkgo fruit extract.

Results

Effect of *Ginkgo biloba* extract on cell growth. The cells were collected and counted after treatment for 48 h. The number of MCF-7 cells in the control, first and second groups was 4.2 $\times 10^6$, 3.3 $\times 10^6$ and 2.6 $\times 10^6$, respectively. The number of MDA-MB-231 cells in the control, first and second groups was 1.3 $\times 10^7$, 0.9 $\times 10^7$ and 0.6 $\times 10^7$, respectively. The cell numbers in the control groups were distinctly higher compared to those in the treatment groups; in addition, the cell numbers in the *Ginkgo biloba* leaves extract-treated group were significantly higher compared to the ginkgo fruit extract-treated group. These results indicated that treatment with *Ginkgo biloba* extract significantly inhibited breast cancer cell proliferation and the ginkgo fruit extract exerted a more potent inhibitory effect compared to the *Ginkgo biloba* leaves extract (Fig. 1).

The cell morphology in the *Ginkgo biloba* extract and the control group were further examined under a confocal laser scanning microscope after treatment for 48 h. As shown in Fig. 1, in the *Ginkgo biloba* leaves extract-treated group and the ginkgo fruit extract-treated group, the cells were prone to grow in clusters compared to the cells in the control group. Moreover, the ginkgo fruit extract group exhibited more extensive cell morphology changes compared to the *Ginkgo biloba*

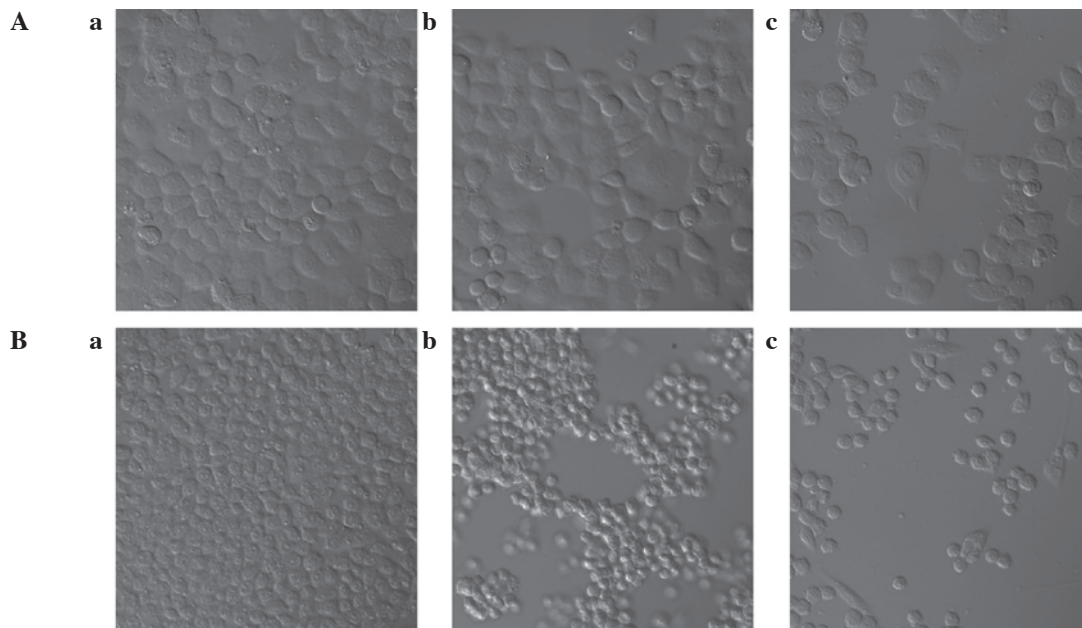


Figure 1. Cell morphology changes in MCF-7 and MDA-MB-231 human breast cancer cell lines treated with the *Ginkgo biloba* extract. (A) Morphological changes in MCF-7 human breast cancer cells: a, control group; b, *Ginkgo biloba* leaves extract-treated group; c, ginkgo fruit extract-treated group. (B) Morphological changes in MDA-MB-231 human breast cancer cells: a, control group; b, *Ginkgo biloba* leaves extract-treated group; c, ginkgo fruit extract-treated group.

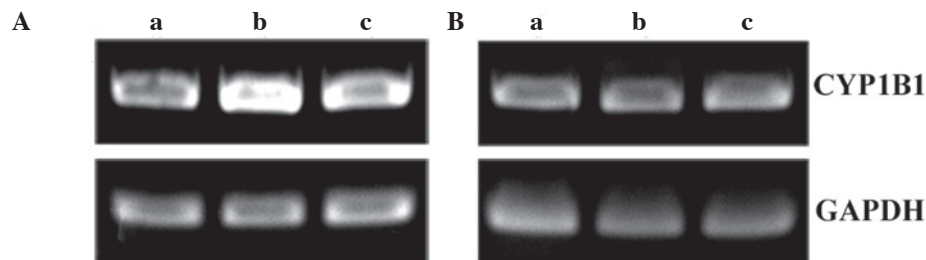


Figure 2. Expression of CYP1B1 in (A) MCF-7 and (B) MDA-MB-231 human breast cancer cell lines treated with the *Ginkgo biloba* extract. The expression of CYP1B1 was analyzed by semi-quantitative reverse transcription-polymerase chain reaction. The GAPDH mRNA was amplified and used as a control. Lanes a and d, control group; b and e, *Ginkgo biloba* leaves extract-treated group; c and f, ginkgo fruit extract-treated group.

leaves extract group, further indicating the more potent growth inhibitory effect of ginkgo fruit extract treatment compared to the *Ginkgo biloba* leaves extract treatment.

Induction of CYP1B1 expression in human breast cancer cells treated with *Ginkgo biloba* extract. The gene expression of CYP1B1 was first confirmed by RT-PCR amplification of the CYP1B1 cDNA (Fig. 2). To ascertain the mechanisms of the inhibition effect of *Ginkgo biloba* extract on breast cancer cell proliferation and growth, the CYP1B1 gene expression was analyzed by qPCR. As shown in Fig. 3, the CYP1B1 expression in the *Ginkgo biloba* leaves extract- and ginkgo fruit extract-treated MDA-MB-231 and MCF-7 cells was upregulated. In MCF-7 cells, the CYP1B1 expression was 1.5- and 1.1-fold higher in the ginkgo fruit extract- and in the *Ginkgo biloba* leaves extract-treated group, respectively, compared to that in the control group. However, in MDA-MB-231 cells, CYP1B1 expression was markedly enhanced, being 7.2- and 2.0-fold higher in the ginkgo fruit extract- and in the *Ginkgo biloba* leaves extract-treated group, respectively, compared to that in the control group.

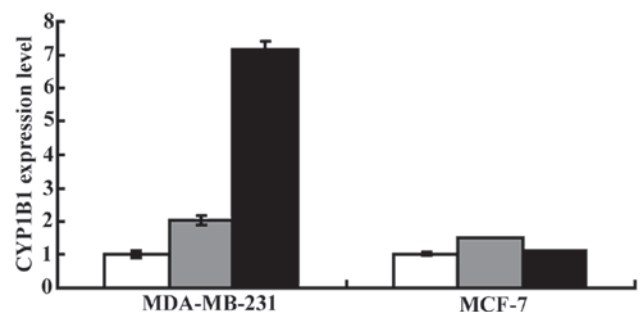


Figure 3. Quantitative polymerase chain reaction analysis of the expression of CYP1B1 at the mRNA level in MCF-7 and MDA-MB-231 cells treated with the *Ginkgo biloba* extract. White columns, expression of CYP1B1 in the control group; grey columns, expression of CYP1B1 in the *Ginkgo biloba* leaves extract-treated group; and black columns, expression of CYP1B1 in the ginkgo fruit extract-treated group.

Collectively, these results demonstrated that treatment with the *Ginkgo biloba* extract enhanced CYP1B1 expression in MDA-MB-231 and MCF-7 cells. The results also demonstrated that MDA-MB-231 cells were more sensitive

to *Ginkgo biloba* extract treatment compared to MCF-7 cells. Moreover, the ginkgo fruit extract treatment resulted in a significantly higher induction of CYP1B1 expression compared to the *Ginkgo biloba* leaves extract, demonstrating the difference in the induction potential of the two different types of *Ginkgo biloba* extracts.

Discussion

In this study, we investigated the treatment effects of the *Ginkgo biloba* leaves extract and ginkgo fruit extract on breast cancer cell proliferation, cell morphology changes and gene expression of CYP1B1. Our results demonstrated that the treatment of MDA-MB-231 human breast cancer cells with either *Ginkgo biloba* leaves extract or ginkgo fruit extract significantly enhanced CYP1B1 gene expression. Moreover, treatment with ginkgo fruit extract resulted in a more significant enhancement of CYP1B1 gene expression in MDA-MB-231 cells. However, the treatment of MCF-7 cells with either *Ginkgo biloba* leaves extract or ginkgo fruit extract did not result in a significant enhancement of CYP1B1 gene expression. Taken together, these results indicate that the ginkgo fruit extract was the most effective inducer of CYP1B1 gene expression in human MDA-MB-231 cells. In addition, MDA-MB-231 cell proliferation was also greatly decreased following treatment with *Ginkgo biloba* leaves extract or ginkgo fruit extract and the greatest decrease was observed in the ginkgo fruit extract treatment group, in parallel with the CYP1B1 gene expression changes.

CYP1B1 is an inducible enzyme regulated through the AhR (33) and is activated by PAHs and dioxin-like compounds (34). CYP1B1 is regulated by several transcription factors, including the AhR/AhR nuclear translocator complex (Ahr/ARNT), the Sp1 transcription factor, the cyclic AMP (cAMP)-response element-binding protein (CREB) and the estrogen receptor (ER). An estrogen-responsive element that is located in close proximity to the Sp1 transcription factor binding site was recently shown to be involved in the ER α regulation of CYP1B1 expression (35). Estrogen is required for the enhanced AhR expression and constitutive induction of CYP1B1 expression in MCF-7 cells (36), indicating the possible involvement of estrogen in the induced expression of CYP1B1 in breast tumor cells and the correlation of CYP1B1 expression with the ER α status (37). In ER-negative breast cancer cells, the presence of estrogen was not able to stimulate the expression of CYP1B1 (35), indicating the dependence of CYP1B1 expression on ER. In addition, tamoxifen treatment upregulated the expression of CYP1B1 in breast cancer cells, suggesting that the induction of CYP1B1 expression may reduce the treatment effects of tamoxifen (38). However, in some cells, the expression of CYP1B1 does not depend on estrogen (39), suggesting that CYP1B1 expression may be independent of estrogen and ER, although it may be induced through alternative pathways. 7,12-Dimethylbenz(a)anthracene-induced carcinogenesis is an extensively investigated model of PAH-induced mouse carcinogenesis and a recent study demonstrated that CYP1B1 knockout mice exhibited resistance to 7,12-dimethylbenz(a)anthracene-induced carcinogenesis (40), demonstrating the metabolic effects of CYP1B1. The results of those studies indicated that the

regulation of CYP1B1 expression is complex, involving transcription factors, ER and AhR.

According to a previous study, treatment of MDA-MB-231 or MCF-7 breast cancer cells with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) inhibited cell growth, accompanied by enhanced expression of CYP1B1 by 8-fold in MCF-7 and 30-fold in MDA-MB-231 cells (41). It was previously demonstrated that in MCF-7 cells, the DNA synthesis was significantly suppressed following treatment with TCDD, suggesting an AhR-independent pathway of TCDD-induced antiproliferation in breast cancer cells (42). Moreover, Larsen *et al* (43) observed that TCDD induced the expression of CYP1B1 mRNA in ER-negative primary human breast epithelial cells, suggesting an alternative pathway of TCDD-induced CYP expression in human breast cancer cells instead of the ER-dependent one. Larsen *et al* (43) also demonstrated that in ER-positive and ER-negative breast cancer cells, CYP1B1 was expressed constitutively and was induced by TCDD (44). Results of the present study have shown that in ER-negative breast cancer cells, the inhibitory effects of *Ginkgo biloba* extract on MDA-MB-231 cell proliferation were correlated with CYP1B1 expression induction, which may occur through an ER and AhR-independent pathway. However, the underlying mechanisms require elucidation through further investigations.

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